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Anti-diabetic effect of isoflavone rich kudzu root extract in experimentally induced diabetic rats

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ABSTRACT

The anti-diabetic effect of isoflavone-rich kudzu extract obtained using natural deep eutectic solvent was assessed in diabetic wistar rats treated at doses of 100 mg/kg or 200 mg/kg for 28 days. Effect of treatment was based on weight, glucose tolerance, fasting glucose and glycated haemoglobin level, hepatic biochemical parameters, expression of anti-insulin and Ki67 antibodies, and histopathological evaluation of pancreas and kidney tissues. We found overall improvement in weight in diabetic rats treated with the extract at dose 200 mg/kg, but not at dose of 100 mg/kg. Plasma level of alanine transaminase (ALT) and aspartate aminotransferase (AST), but not alkaline phosphatase (ALP), were significantly different between untreated diabetic rats and those treated with the extract ($p = 0.05$). We found that the isoflavones-rich extract stimulated β-cell regeneration in a dose-dependent effect and showed no observable toxic impact in either the kidney or pancreas. Thus, this extract may be a promising treatment option for diabetic condition.

1. Introduction

Diabetes mellitus (DM) is a metabolic disease plaguing a substantial number of people, with a global estimate of approximately 424.9 million people living with this disease in 2017 and expected upsurge to 628.6 million by 2045, these staggering figures makes this disease one of the leading global hospital burden (IDF, 2017). DM can be chiefly divided into two main types, type I diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). While T1DM is a disease characterized by the total destruction of pancreatic β-cells, T2DM is marked by dysfunctional pancreatic β-cells and insulin resistance (Aguayo-Mazzucato & Bonner-Weir, 2018). Pancreatic β-cell function and mass play critical role in the manifestation of this disease, thus the regeneration of lost functional β-cell mass offers therapeutic strategy to alleviate the progression of this disease (Aguayo-Mazzucato & Bonner-Weir, 2018). While the complexity of DM pathogenesis cannot be overstated, this disease is characterized by hyperglycemia, a condition that could possibly progress to advance consequences such as macrovascular complications (coronary artery disease and peripheral arterial disease), and microvascular complications (diabetic retinopathy, nephropathy, and neuropathy) when left untreated (Fowler, 2008). Therefore, lowering glucose level is one of the pivots in the management of diabetes (Fowler, 2008). Diabetic nephropathy (DN) has been highlighted as the leading cause of end stage renal disease (ESRD), a condition which could be fatal when left uncontrolled (Yaribeygi, Mohammadi, Rezaee, & Sahebkar, 2018). The onset and progression of DN is marked by functional and structural changes in kidney which include an increase in albumin level, glomerular hyperfiltration, tubular atrophy, and arteriolar hyalinosis (Lim, 2014). Advance consequences have plagued the long-term use of most antidiabetic therapies used in clinical practice, hence, the need for sustainable solution remains a major concern (Wareham & Herman, 2016). In preclinical studies, different chemicals have been successfully used to induced diabetes, one of which is alloxan monohydrate, this compound has been found to generate reactive

Abbreviations: DM, diabetes mellitus; DN, diabetic nephropathy; ESRD, end stage renal disease; ROS, reactive oxygen species; NADES, natural deep eutectic solvents; DL, detection limit; QL, quantitation limit; OGTT, oral glucose tolerance test; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; DAB, diaminobenzidine; STZ, streptozotocin; WBC, white blood cell; MPV, mean platelet volume; PDW, platelet distribution width; PCT, plateletcrit; PLT, platelet; PAS, Periodic acid–Schiff

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oxygen species (ROS) which in turn results to the necrotic death of pancreatic β-cells (Radenkovic, Stojanovic, & Prostan, 2016).

Dietary phytoestrogens, especially isoflavones, have been on the limelight as potential antidiabetic therapeutics owing to their ability to scavenge free radical, prevent inflammation, and lower blood glucose level (Duru, Kovaleva, Danilova, Van der Bijl, & Belousova, 2018; Lee, 2006). Isoflavones are phytochemicals richly found in leguminous plants (such as *Glycine* max and *Pueraria lobata*), these phytochemicals share structural similarity with the human estrogen, thus exhibiting potential health benefits by acting on estrogen receptors (Bhathena & Velasquez, 2002). Chemical analysis of *Pueraria lobata* (popularly known as kudzu) extract identified 3 main isoflavones namely puerarin, daidzein, and genistein (Bebrevska, Theunis, Vlietinck, Pieters, & Apers, 2008). This extract has been widely used in Chinese traditional medicine to treat diseases such as diarrhoea, diabetes, and cardiovascular diseases (Wong, Li, Li, Razmovski-Naumovski, & Chan, 2011).

While most extraction techniques of isoflavones from raw sources have been performed using conventional organic solvents which are perceived to be non-environmentally friendly and toxic, the need to develop green extraction solvents remains critical for the broad use of isoflavones in pharmaceuticals (Bajkacz & Adamek, 2017). Natural deep eutectic solvents (NADES), a class of green solvents that are synthesized from natural metabolites, have shown promising advantages over conventional solvents such as non-toxicity and biodegradability (Choi et al., 2011). While these solvents have been applied in the extraction of different bioactive compounds, only few studies have succeeded in extracting isoflavones using them (Bajkacz & Adamek, 2017; Nam, Zhao, Lee, Jeong, & Lee, 2015).

This study investigates the antidiabetic properties of isoflavone-rich extract obtained from kudzu roots using NADES in alloxan induced diabetic rats. Evaluation of the antidiabetic properties of the isoflavone-rich extract was based on key parameters such as glycemic control, haematological indices, hepatic enzyme activities, and regeneration of pancreatic β-cells. Furthermore, we explored the renal protective or possible nephrotoxicity effect of the isoflavone-rich extract in diabetic and healthy rats.

2. Materials and method

2.1. Materials

Daidzein, genistein, puerarin, vitexin, and 4-hydroxyflavanone standards were purchased from Sigma Aldrich (Missouri, USA). Dried kudzu roots were purchased from Xi'an Sgonek Biological Technology (Shaanxi Sheng, China). Alloxan monohydrate was purchased from Sigma Aldrich (Missouri, USA).

2.2. Preparation of natural deep eutectic solvent (NADES)

NADES comprising choline chloride and citric acid (1:2, mol/mol) was prepared as described by Dai, Spronsen, Witkamp, Verpoorte, and Choi (2013). Briefly, the calculated amount of choline chloride and citric acid was transferred into a glass seal and 20% of distilled water was added to the mixture. The mixture was heated at 60–80 °C under constant stirring until a transparent solution was observed. The solution was stored in dark until when used.

2.2.1. Extraction and quantification of isoflavones from kudzu roots

Extraction of isoflavones from dried kudzu root was carried out using NADES at a solvent volume (mL) to material ratio (g) of 20:1. Extraction was done in an ultrasonic bath at 50 °C for 3 h. The extract was separated from the insoluble fractions by centrifugation (6000 rpm for 30 mins), then concentrated to dryness using a rotary evaporator and dissolved in methanol prior HPLC analysis. Isoflavone content of the extract was analyzed as described by Bajkacz and Adamek (2017) using an Agilent Zorbax SB-C18 (150 mm × 2.1 mm) column on the Agilent 1100 series liquid chromatograph (consisting a pump, degasser, autosampler, and UV/Vis detector). Samples were injected at a volume of 250 µL. The binary mobile phase comprised water +2 mL/L formic acid (solvent A) and acetonitrile (solvent B). A linear gradient elution of solvent B was applied starting from 15% to 85% over 20 mins, at a flow rate of 0.3 mL/min. The temperature of the column was kept at 35 °C. Isoflavones were separated on a reversed stationary phase and detected with UV at 254 nm. Daidzein, puerarin, and genistein were used as standards, while vitexin, and 4-hydroxyflavanone were used as external standards. Comparison of the retention and UV spectra of each individual isoflavone contained in the extract with those of pure standards was used for identification. Quantification of each isoflavone content in the extract was done using calibration curves of average peak areas of the standard solutions against the corresponding concentrations as described by Kao and Chen (2002). The detection limit (DL) and quantitation limit (QL) was determined in accordance to the method described by the ICH (1996).

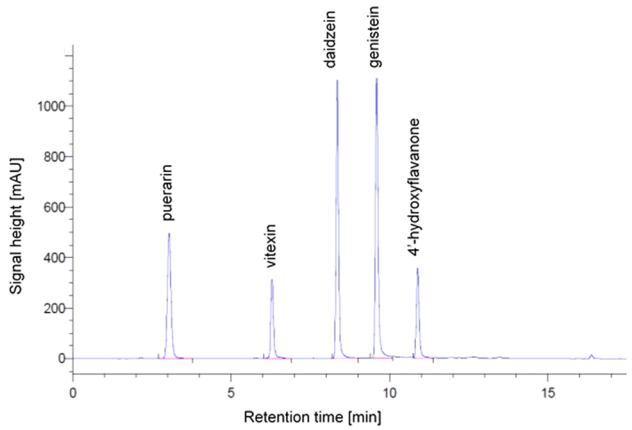


Fig. 1a. Shows HPLC chromatogram of showing peaks of isoflavone standards.

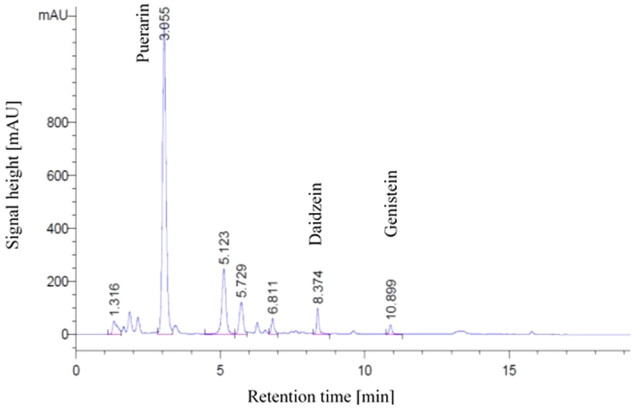


Fig. 1b. Shows HPLC chromatogram of Isoflavone-rich extract obtained from kudzu using NADES extract of kudzu root.

2.3. Experimental animals

Female Wistar rats (12-weeks old) of weight 205 ± 7 g showing no disease symptoms were procured from the animal facility of the Ural Branch of Russia Academy of Science (UB RAS) and used for the study. The Animals were housed 5 per polypropylene cage under standard laboratory conditions, 20 ± 2 °C, light: dark cycle (12 h:12 h). Rats were fed with standard rat chow (Teklad 2016, Teklad diets, Madison, WI, USA; 16.4% protein, 4.0% fat, 3.3% crude fibre, and 48.5% carbohydrate) and allowed free access to purified water. All the animal experiment protocols were approved by the Animal Ethics Committee of the Ural Branch of Russia Academy of Science (UB RAS). Rats were

Table 1
Effect of isoflavone rich extract on body weight of experimental animals.

Experimental group	7 days	14 days	21 days	28 days
Control rats + vehicle (NC)	274 ± 14*	303 ± 15*	317 ± 17*	320 ± 17*
Control rats + isoflavone-rich kudzu root extract (100 mg/kg) (NC + Iso)	295 ± 27*[a]	311 ± 27*[a]	321 ± 29*[a]	327 ± 26*[a]
Control rats + isoflavone-rich kudzu root extract (200 mg/kg) (NC + Iso 200)	306 ± 26*[a]	327 ± 17*[a]	327 ± 14*[a]	331 ± 11*[a]
Diabetic control + vehicle (DM)	208 ± 10	200 ± 13	193 ± 5	190 ± 8
Diabetic + isoflavone-rich kudzu root extract (100 mg/kg) (DM + Iso)	208 ± 8	222 ± 21	222 ± 22	227 ± 17*
Diabetic + isoflavone-rich kudzu root extract (200 mg/kg) (DM + Iso 200)	217 ± 16	238 ± 22*	253 ± 19*	253 ± 19*

Data are shown as mean ± SD.
* Indicates significant difference compared to DM group in the same column ($p < 0.05$).
[a] Indicates no significant difference compared to NC group ($p = 0.05$).

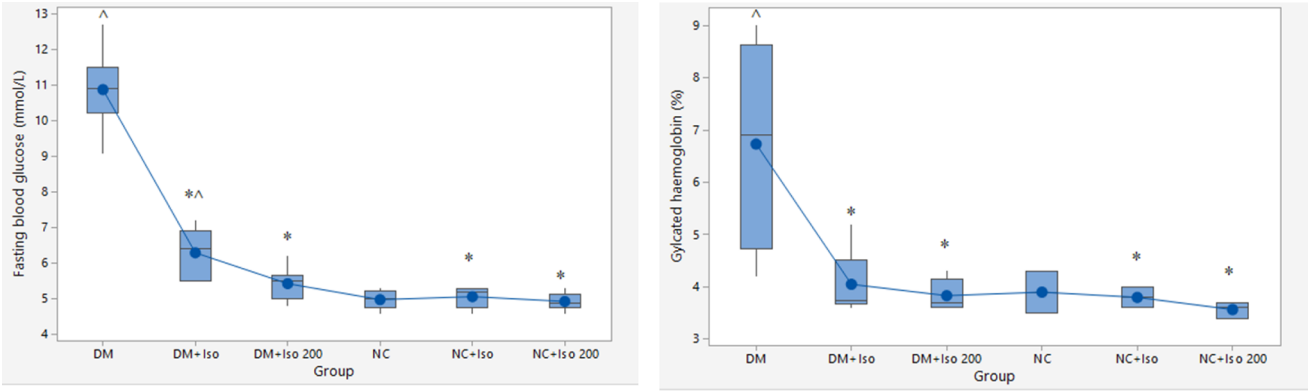


Fig. 2. Depicts the effect of isoflavone-rich kudzu extract treatment in diabetic and healthy rats. Blood concentrations of (a): fasting glucose, (b): glycated haemoglobin (HbA1c). *indicates significant difference compared to DM group ($p < 0.01$); ^ indicates significant difference compared to NC group ($p < 0.01$).

Table 2
Effect of the isoflavone-rich extract on insulin level and oral glucose tolerance in diabetic animals.

Experimental group	Blood glucose levels (mmol/L)				AUC mmol/L/h
	0 min	30 mins	60 mins	120 mins	
Control rats + vehicle (NC)	4.9 ± 0.47	7.5 ± 0.32	5.7 ± 0.45	5.1 ± 0.51	11.8 ± 0.87
Diabetic control + vehicle (DM)	9.4 ± 1.17[a]	14.5 ± 0.76 [a]	12.3 ± 0.56[a]	12 ± 0.92[a]	24.8 ± 1.44[a]
Diabetic + isoflavone-rich kudzu root extract (100 mg/kg) (DM + Iso)	6.3 ± 0.95*	9.3 ± 0.47*[a]	7.7 ± 1.15*[a]	6.7 ± 0.67*	15.4 ± 1.67*[a]
Diabetic + isoflavone-rich kudzu root extract (200 mg/kg) (DM + Iso 200)	5.4 ± 0.47*	8.5 ± 0.32*	6.2 ± 0.45*	5.7 ± 0.51*	13.1 ± 0.9*

Data are shown as mean ± SD.
[a] Shows significant difference compared to NC group ($p < 0.01$).
* Shows significant difference compared to DM group ($p < 0.01$).

Table 3
Effect of isoflavone extract on liver and renal parameters.

Experimental group	AST (U/L)	ALT(U/L)	ALP(U/L)	Total protein (g/L)	Urea (mmol/L)
Control rats + vehicle (NC)	13.4 ± 1	9 ± 2.2	51.3 ± 5.3[b]	69.5 ± 3.1	5.03 ± 0.06
Control rats + isoflavone-rich kudzu root extract (100 mg/kg) (NC + Iso)	15.2 ± 4.2*	8.6 ± 6.2*	35.6 ± 8.8[b]	72.9 ± 3.6*	3.6 ± 0.6*
Control rats + isoflavone-rich kudzu root extract (200 mg/kg) (NC + Iso)	15.3 ± 1.3*	7.2 ± 2.1*	34.7 ± 7.9[b]	73.6 ± 6.4*	3.1 ± 0.3*[a]
Diabetic control + vehicle (DM)	21 ± 2.4[a]	16.3 ± 2.6 [a]	63.4 ± 26.4[b]	59.1 ± 3.9 [a]	8.2 ± 1.2 [a]
Diabetic + isoflavone-rich kudzu root extract (100 mg/kg) (DM + Iso)	15.6 ± 1.74*	8.3 ± 1.9*	62.8 ± 4[b]	73.0 ± 3.5*	4.8 ± 0.3*
Diabetic + isoflavone-rich kudzu root extract (200 mg/kg) (DM + Iso 200)	14.9 ± 2.1*	7.9 ± 1.4*	58.7 ± 6.5[b]	71.5 ± 2.9*	4.9 ± 0.6*

Data are shown as mean ± SD.
[a] Shows significant difference compared NC group ($p < 0.01$).
* Shows significant difference compared to DM group ($p < 0.01$).
[b] Shows no significant difference between groups ($p \geq 0.05$).

Weight of rats was recorded weekly during the study.

2.4. Induction of diabetic and grouping

Diabetes was induced by a single intraperitoneal (i.p) injection of freshly prepared alloxan monohydrate (170 mg/kg) after 16 h of fasting. Prior injection, alloxan monohydrate was dissolved in 10 mM sodium citrate (pH 4.5). Fasting blood glucose (FBG) level was measured 72 h after injection and rats with FBG level of ≥7.1 mmol/L were selected as successfully prepared diabetic models and used in the study.

Rats were randomly divided into six groups, each group comprised ten ($n = 10$) rats. The groups are as follows: NC group: healthy control

Table 4
Influence of isoflavone rich kudzu root extracts on hematological parameters.

Parameters	Control rats + vehicle (NC)	Control rats + isoflavone-rich kudzu root extract (100 mg/kg) (NC + Iso)	Control rats + isoflavone-rich kudzu root extract (200 mg/kg) (NC + Iso)	Diabetic control + vehicle (DM)	Diabetic + isoflavone-rich kudzu root extract (100 mg/kg) (DM + Iso)	Diabetic + isoflavone-rich kudzu root extract (200 mg/kg) (DM + Iso 200)
MPV (f/L)	6.6 ± 0.1[b]	6.4 ± 0.3[b]	6.4 ± 0.1[b]	6.6 ± 0.2[b]	6.5 ± 0.1[b]	6.4 ± 0.2[b]
PCT (%)	0.5 ± 0.01	0.4 ± 0.03[*]	0.45 ± 0.05[*]	0.6 ± 0.06 [a]	0.5 ± 0.06	0.5 ± 0.07
PLT($\times 10^3$/UL)	753 ± 19.2[*]	588 ± 65.6[*]	703 ± 67.1[*]	948 ± 106.8[a]	841 ± 101.9	762 ± 18.9
PDW(f/L)	11.2 ± 0.4[b]	11 ± 0.2[b]	10.7 ± 0.3[b]	11.2 ± 0.3[b]	11.2 ± 0.2[b]	11.1 ± 0.3[b]
WBC	6.7 ± 0.7[b]	7.5 ± 0.5[b]	7.6 ± 0.9[b]	12.8 ± 5.7[b]	7.8 ± 3.2[b]	8.1 ± 2.9[b]

Data are shown as mean ± SD.
[a] Shows significant difference compared NC group ($p < 0.01$).
[*] Shows significant difference compared to DM group ($p < 0.01$).
[b] Shows no significant difference between groups ($p \geq 0.05$).

rats + vehicle (distilled water), NC + Iso group: healthy control rats + isoflavone-rich kudzu root extract (100 mg/kg), NC + Iso 200 group: healthy control rats + isoflavone-rich kudzu root extract (200 mg/kg), DM group: diabetic control rats + vehicle , DM + 1so group: diabetic rats + isoflavone-rich kudzu root extract (100 mg/kg), DM + 1so 200 group: diabetic rats + isoflavone-rich kudzu root extract (200 mg/kg). The isoflavone-rich extract was dissolved in distilled water and administered via oral gavage; animals were treated thrice weekly during the 28 experimental days. Treatment of rats in each classified group was initiated 4 days after alloxan monohydrate injection.

Fasting blood glucose, glycosylated hemoglobin (HbA1c), and oral glucose tolerance test (OGTT)

At the end of the study, all rats were fasted, and blood was collected via cardiac puncture. FBG level was determined using glucose oxidase method as described by Danilova et al. (2017), and glycosylated hemoglobin (HbA1c) measured using affinity chromatography (TOR 9398240-16404416-01, Fosfosorb OJSC, Russian Federation) according to Jeppsson et al. (2002). Assessment of oral glucose tolerance test (OGTT) of rats in NC, DM and DM + Iso, and DM + Iso 200 group, was done using a OneTouch glucometer (LifeScan, Wayne, PA, USA. This assessment was based on the FBG level at 0 min, and postprandial glucose (PG) level measured after oral administration of glucose (2 g/kg) at 30, 60, and 120 min. The area under the curve (AUC) were calculated according to Sakaguchi et al. (2016) using the trapezoidal approximation of PG levels.

2.5. Evaluation of hepatic and renal parameters

Plasma level of ALT, AST, ALP, urea, and total protein were determined using ready to use reagents kits (Vital diagnostics, St. Petersburg) as described by Ostroushko et al. (2013). Tests were conducted using a DU-800 spectrophotometer (Beckman Coulter Int. S.A., Switzerland) at specified wavelength according to manufacturers.

2.6. Determination of haematological parameters

White blood cell (WBC), Mean platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT), and platelet count (PLT) were estimated using automated haematology analyzer (Biocode Hycel, France) with heparinized blood samples.

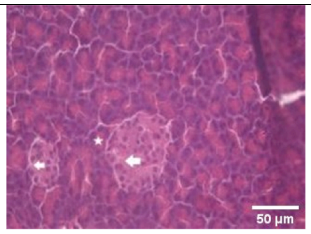
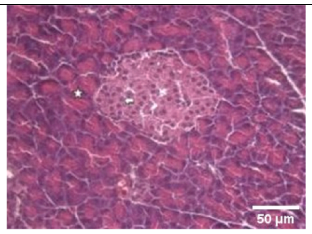
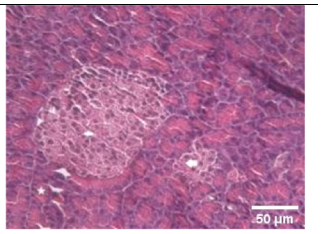
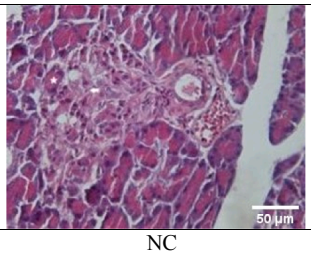
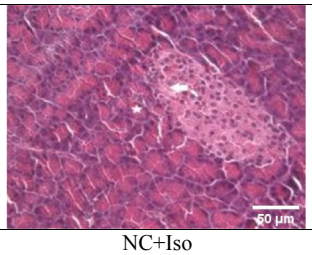
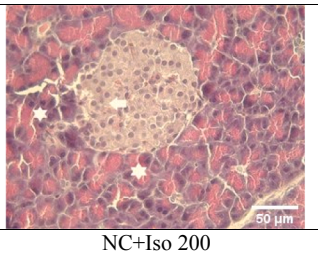
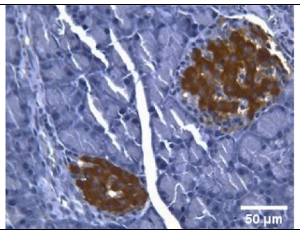
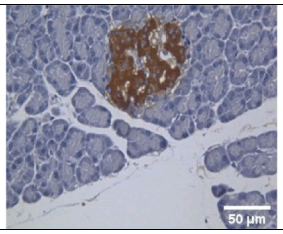
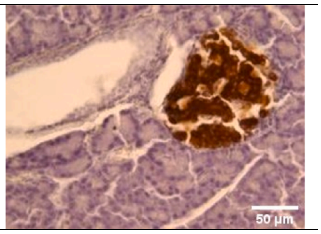
2.7. Histological and immunohistochemical study

Rats were euthanized by cervical dislocation, and the kidney and pancreas were excised and fixed in 10% formalin. Tissues were processed using a tissue processor (Leica TP 1020) and embedded in paraffin blocks. Approximately 3–4 μm sections of kidney and pancreas tissue were stained with hematoxylin and eosin (H&E) dye, kidney tissue sections were additionally stained with periodic acid schiff (PAS) dye.

Immunohistochemical detection of anti-insulin and Ki67 antibodies in pancreatic tissues was done as per the avidin–biotin peroxidase complex (ABC) method. Briefly, tissue sections were deparaffinized and incubated in antigen retrieval buffer (0.01 M citrate buffer, pH 6.0) for 15 min at 95 °C. Afterwards, tissue sections were incubated with primary antibody for insulin (clon E11D7, Millipore; diluted at 1:200) or Ki67 (Leica, Biosystems; diluted at 1:25), overnight at 4 °C. Tissue sections were further incubated in Biotinylated secondary antibody for 1 h at room temperature. All tissue sections were examined under light microscope (Leica DM 2500) using ×40 magnification objective.

2.8. Morphometric analysis

The total number of islets and number of islets showing positive insulin response in 1 mm^2 of the pancreatic parenchyma (N/mm^2) were

	NC	NC+Iso	NC+Iso 200
(a) H&E			
	DM	DM+Iso	DM+Iso 200
			
	NC	NC+Iso	NC+Iso 200
(b) Insulin			
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	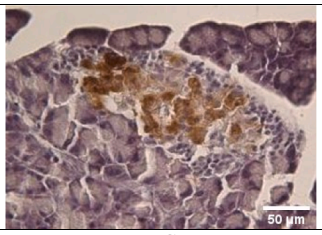	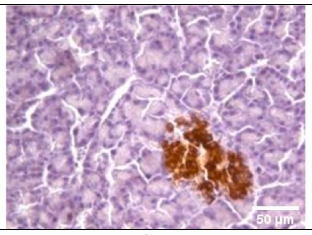	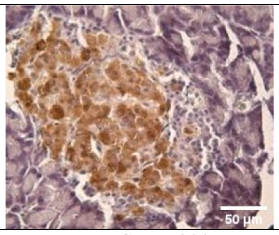
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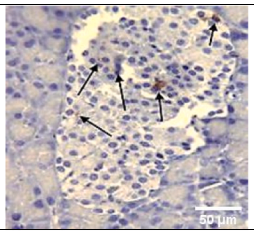
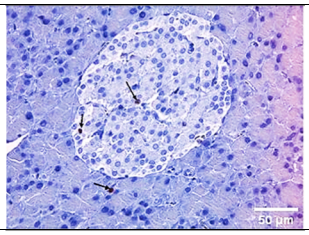
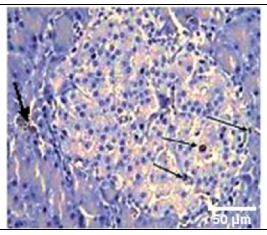
(C) Ki67			
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	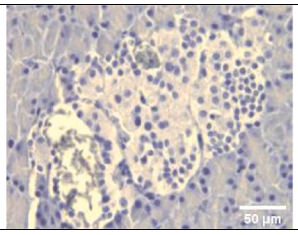	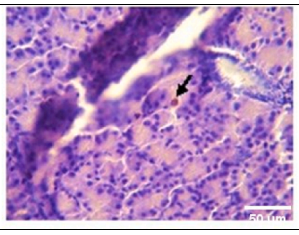	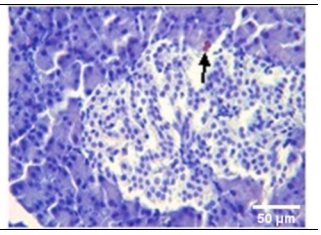

(caption on next page)

Fig. 3. Light microscopic pictures of pancreas sections. (a) Hematoxylin and eosin staining ×400; NC group showing normal structure of the Islets of Langerhans (arrow) and acini(star); NC + Iso group showing normal structure of the Islets of Langerhans (arrow) and acini(star); NC + Iso 200 group showing normal structure of the Islets of Langerhans (arrow) and acini(star); DM group showing degeneration of the islet (arrow); DM + Iso group showing preservation of the architectural structure of Islet of Langerhans (arrow); DM + Iso 200 group showing restoration of the architectural structure of Islet of Langerhans (arrow), acini (star). (b) Insulin immunostaining (brown colour) ×400; NC group showing intense insulin-positive β cells; NC + Iso group showing high number of insulin positive β cells (brown colour); NC + Iso200 group showing high intense insulin positive β cells (brown colour); DM group showing a decreased number of insulin-positive β cells; DM + Iso and DM + Iso 200 group showing an increased number of insulin-positive β cells as compared to the DM group. (c) Ki67 immunostaining ×400, ki67-positive cells are identified by their brown colour (arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

counted. We estimated the percentage of β-cells in each individual islet based on the total number of islet cells and those showing positive insulin staining (brown stain). The insulin content in β-cells was assessed based on the optical density of the stained cells using the image analysis software Video Test "Morphology" 5.0. All morphometric evaluations were based on 20–30 microscopy field per tissue section of each rats.

2.9. Statistical analysis

Statistical analysis was performed using Statistica 6.0 (StatSoft, Inc). Data were presented as mean ± SD based on 3 independent measurements, and significant difference between group was considered at $P < 0.05$ or 0.01. Data were analyzed by one-way ANOVA and Dunnett's T test.

3. Results

3.1. Identification and quantification of isoflavones content in kudzu root extract

The HPLC chromatogram showing peaks of isoflavone standards used in the current study was presented in Fig. 1a. The HPLC analysis showed that the obtained kudzu root extract contains daidzein, genistein, and puerarin (Fig. 1b). Quantification of the individual isoflavone content of the extract showed that the extract contained 3.69 ± 0.02% puerarin (36.9 mg/g of extract), 0.27 ± 0.005% daidzein (2.7 mg/g of extract), and 0.037 ± 0.001% genistein (0.4 mg/g of extract), respectively. This implied that 2.5 g and 5 g of the extract contained 100 mg and 200 mg of total isoflavones, respectively.

3.2. Effect of isoflavone-rich kudzu root extract on body weight

As shown in Table 1, an approximately 8.7% decrease in weight was observed in the DM group during the study, this weight loss was ameliorated in diabetic rats treated with the isoflavone-rich extract at dose of 200 mg/kg. However, this could not be said when diabetic rats treated with the isoflavone-rich extract at dose of 100 mg/kg as no significant improvement in weight was observed, not until the 4th experimental week. As presented in Table 1, we found no significant difference ($p = 0.05$) between weight of healthy control rats (NC group) and healthy rats treated with the isoflavone-rich extract (NC + Iso and NC + Iso 200 group)

3.3. Influence of the isoflavone-rich kudzu root extract on blood glucose, oral glucose tolerance, and glycated haemoglobin

As depicted in Fig. 2a, we found that the isoflavone-rich kudzu extract had no advance effect on glycemic control as FBG level measured in healthy control rats (NC group) and healthy rats treated with the isoflavones-rich extract (NC + Iso and NC + Iso 200 group) was not significantly different ($p = 0.05$) . As illustrated in Fig. 2a, FBG level was significantly elevated in diabetic rats compared to healthy control rats, this elevated level was found to decrease in a dose-dependent manner with the administration of the isoflavone-rich extract , with a significant difference only observed at a treatment dose of 200 mg/kg (DM + Iso 200 group). Similarly, as presented in Fig. 2b, a pronounced elevation in HbA1c level was observed in DM group compare with NC group ($p < 0.05$), this was also significantly decreased in a dose-dependent manner in with the isoflavone-rich extract treatment. We observed no significant difference in HbA1c levels between NC, NC + Iso, and NC + Iso 200 group ($p = 0.05$).

As shown in Table 2, diabetic rats showed poor glucose tolerance, this was visible from higher AUC value observed in DM group as compared to NC group ($p < 0.05$). When compared to DM group, DM + Iso and DM + Iso 200 group showed improved glucose tolerance, this was evident from their lower AUC values (Table 2). We observed that the AUC value of NC group and DM+Iso 200 did not significantly differ ($p = 0.05$).

3.3.1. Effect of isoflavone rich kudzu root extract on hepatic and renal biochemical parameters

As presented in Table 3, there was increase in the serum level of AST and ALT in DM group compare with NC group ($p < 0.05$). The treatment of diabetic rats with the isoflavone-rich extract lowered the AST and ALT levels (Table 3). No significant difference was observed in serum ALP level in rats of all experimental groups ($p = 0.05$).

As shown in Table 3, a marked decrease in total protein and increase in serum urea level was observed in DM group as compared to NC group ($p < 0.05$). The treatment of diabetic animals with the isoflavone-rich extract improved total protein level, from 59.1 ± 3.9 g/L observed in DM group to 73.0 ± 3.5 and 71.5 ± 2.9 in DM + Iso and DM + Iso 200, respectively (Table 3). The treatment of diabetic rats with the isoflavone-rich extract significantly lowered the serum urea level from 8.2 ± 1.2 mmol/L observed in DM group to 4.8 ± 0.3 mmol/L and 4.9 ± 0.6 mmol/L in DM + Iso and DM + Iso 200, respectively (Table 3).

3.4. Influence of isoflavone rich extract on haematological parameters

Data presented in Table 4 show no significant difference in WBC, MPV, and PDW level between the experiment groups ($p = 0.05$). However, an elevated PLT count and PCT level was observed in DM group as compared to NC group ($p < 0.05$). This elevated PLT count was found to be significantly lower ($p < 0.05$) in diabetic rats treated with the isoflavone-rich extract at doses of 200 mg/kg (DM + Iso 200 group). No significant difference was observed between the PLT count in DM group and DM + Iso group ($p = 0.05$). The treatment of diabetic rats with the isoflavone-rich extract showed no effect on the PCT level as no significant difference was observed with treatment at both doses ($p = 0.05$). The level of PCT and PLT count was insignificantly different between NC, NC + Iso, and NC + Iso200 group (Table 4).

3.5. Histological evaluation of pancreatic islets

Normal distribution of noncapsulated, oval or rounded pancreatic islets with well-defined boundaries was observed in NC + Iso and NC + Iso 200 group (Fig. 3a), which were histological similar to the pancreatic tissues observed in NC group. As presented in Fig. 3a, irregular shaped pancreatic islets with pronounced reduction was observed in DM group, these structural alterations were ameliorated in diabetic rats treated with isoflavone-rich extract (DM + Iso and DM + Iso 200 group). It could be suggested that the treatment with the isoflavone-rich

Table 5
Morphometric analysis of the pancreatic islets of rat.

Parameters	Control rats + vehicle (NC)	Control rats + isoflavone-rich kudzu root extract (100 mg/kg) (NC + Iso)	Control rats + isoflavone-rich kudzu root extract (200 mg/kg) (NC + Iso)	Diabetic control + vehicle (DM)	Diabetic + isoflavone-rich kudzu root extract (100 mg/kg) (DM + Iso)	Diabetic + isoflavone-rich kudzu root extract (200 mg/kg) (DM + Iso)
% of pancreatic islets with positive insulin staining	100 ± 0	100 ± 0*	100 ± 0*	45 ± 6[a]	92 ± 3*[a]	95 ± 2*
Number of pancreatic islets, N/mm2	4.2 ± 1.3	3.9 ± 0.5*	4.1 ± 0.9*	1.6 ± 0.4[a]	1.6 ± 0.5*[a]	2.6 ± 0.4*
% of cells positive to insulin in the pancreatic islets.	77.7 ± 2	77.4 ± 7.4*	78.9 ± 7.4*	47.4 ± 12[a]	62.8 ± 11.8*[a]	68.8 ± 9.1*
Optical intensity of insulin positive cell, standard units/cell	0.4 ± 0.09[b]	0.4 ± 0.09[b]	0.4 ± 0.07[b]	0.3 ± 0.034[b]	0.3 ± 0.04[b]	0.3 ± 0.04[b]
Number of positive Ki67 cells per mm2 of the islet	698.65 ± 15.3	795.38 ± 39.6*[a]	799.18 ± 28.3*[a]	360.88 ± 19.34[a]	400.38 ± 39.6[a]	470.38 ± 19.8*[a]

Data are shown as mean ± SD.
[a] Shows significant difference compared NC group ($p < 0.01$).
[*] Indicates significant difference compared to DM group ($p < 0.01$).
[b] Shows no significant difference between groups ($p \geq 0.05$).

extract somehow prevented further degradation of the pancreatic islets after the induction diabetes.

3.6. Immunohistochemical and morphometric analysis

Based on the data presented in Table 5 and Fig. 3b, we observed that when compared to NC group, DM group showed markedly reduction in percentages of islets with insulin positive response and β-cells per total number of islet cells ($p < 0.05$), the number of pancreatic islets per field (N/mm^2) was also decreased. No significant difference was observed between NC, NC + Iso and NC + Iso 200 group based on the number of pancreatic islets per field (N/mm^2), percentages of islets with positive insulin response and β-cells per total islet cells (Table 5, Fig. 3b). Although no significant difference was observed between DM and DM + Iso group in terms of number of pancreatic islets per field (N/mm^2) ($p = 0.05$) , but at treatment dose of 200 mg/kg (DM + Iso 200 group), the isoflavone-rich extract was found to enhance the number of pancreatic islets per field (N/mm^2) (Table 5).

Also, the treatment of diabetic animal with the isoflavone-rich extract (DM + Iso and DM + Iso 200 group) increased the number pancreatic β -cells compared with DM group (Table 5 and Fig. 3b). The percentage of β-cells per the total number of islet cells was found to increase from 47.4 ± 12% observed in DM group to 62.8 ± 11.8 and 68.8 ± 9.1 in DM and DM + Iso group, respectively. The percentage of pancreatic islets with positive insulin response was also enhanced in diabetic animals treated with the isoflavone extract in a dose-dependent manner with 95 ± 2% and 92 ± 3% of insulin positive pancreatic islet recorded in DM + Iso 200 and DM + Iso, respectively. Interestingly, no difference was observed in the insulin content in β -cells in all groups as the intensity of immunostained insulin positive pancreatic cells between did not differ in all experimental groups ($p = 0.05$).

We noticed that the treatment of normal control animals with the isoflavone-rich kudzu extract at both doses (NC + Iso and NC + Iso 200 group) significantly increased the number of Ki67 positive cells in the pancreatic islets as compared to normal control animals (Fig. 3c, Table 5). An observable decrease in the number of Ki67 positive cells was found in diabetic group which was significantly enhanced by isoflavone-rich kudzu extract treatment at dose of 200 mg/kg (Fig. 3c, Table 5). Although the treatment of diabetic rat with the isoflavone-rich kudzu extract at dose of 100 mg/kg increased the expression of Ki67 positive cells in the pancreatic islets, no significant difference was found compared to DM group (Fig. 3c, Table 5).

3.7. Histopathological evaluation of kidney sections of experimental groups

As illustrated in Fig. 4, abnormal glomerular capillary network and hypercellularity was observed in kidney sections from DM group compared with NC group. These alterations were remediated in diabetic rats treated with the isoflavone-rich extract (DM + Iso and DM + Iso 200 group), as decrease in the cellularity of the glomerulus (the number of mesangial, podocytes, and endothelial cells), and restoration of the histological structure of the glomerular capillary network, were observed (Fig. 4). Furthermore, the treatment of healthy rats with the isoflavone-rich extract (NC + Iso, NC + Iso 200 group) showed normal cellularity and histological structure of glomerulus, similar to those observed in NC group (data not shown). As depicted in Fig. 5, normal cubical simple epithelium cells and brush border of the renal tubules was observed in NC group which were found to be mildly damaged (damage to the proximal convoluted tubules) in DM group. The treatment of healthy rats with the isoflavone-rich extract at both doses showed no nephrotoxic effect, nor alteration of the renal tubules (Fig. 5).

4. Discussion

The antidiabetic potential of the isoflavone-rich extract was

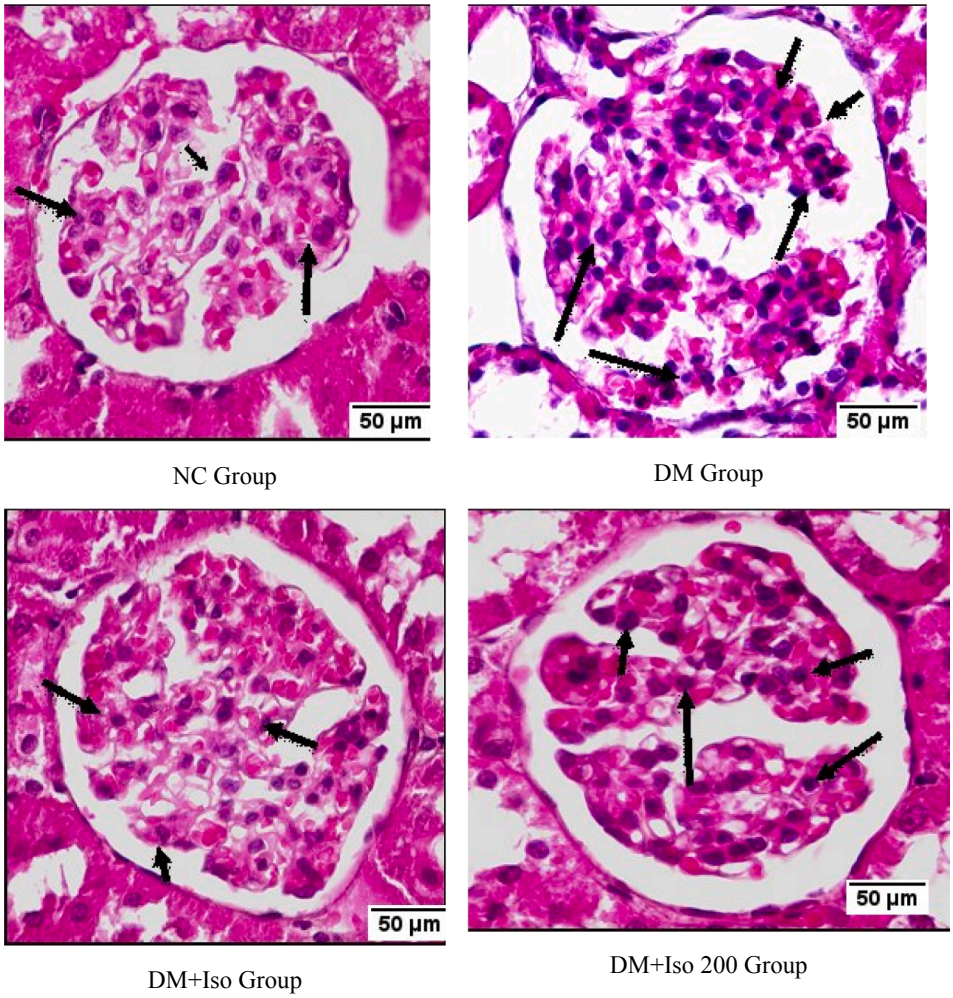

Fig. 4. Showing the effect of isoflavone-rich kudzu extract on histological changes in the glomerulus (a): NC group showing normal glomerular capillary network with podocytes and mesangial cells (arrow); (b): DM group showing hypercellularity of glomerulus, and mesangial cells proliferation (arrow); (c): DM + Iso group showing normal structure of glomerular capillary network with normal cellularity of the glomeruls (arrow); (d): DM + Iso 200 group showing normalcellularity of glomerulus (arrow).

assessed based on effect on glycemic control, liver biomarkers, haematological indices, and expression of insulin and Ki-67 antibody. We observed that the isoflavone-rich extract can potentially improve glycemic control and stimulate the regeneration of pancreatic β-cells, in a dose-dependent manner. The treatment of diabetic rats with the isoflavone-rich extract also ameliorated abnormal circulating level of liver biomarkers (such as AST, ALT, ALP, and Urea) associated with diabetes pathogenesis. Overall, we found that the isoflavone-rich extract had no effect on haematological parameters. Additionally, at both treatment doses, the isoflavone-rich extract did not show any characteristic nephrotoxic effect in healthy rats, rather it exhibited renal protective effect when administered to diabetic rats.

Based on HPLC analysis, we identified three types of isoflavones (puerarin, daidzein and genistein) in the isoflavone-rich kudzu root extract. The isoflavone having the highest content in the isoflavone-rich extract was puerarin (3.69 ± 0.02%) and the least was genistein (0.037 ± 0.001%). This finding supports previous study where puerarin and daidzein were identified in extract obtained from kudzu root, and the same authors found the obtained extract to contain higher percentage of puerarin (Kitada, Mizobuchi, Ueda, & Nakazawa, 1985).

We observed progressive weight loss in DM group. Our finding supports previous study where pronounced decrease in body weight has been reported in diabetic animals (Oza & Kulkarnia, 2018a). The treatment of diabetic rats with the isoflavone-rich extract at dose of 200 mg/kg, improved body weight starting from the 2nd experimental week. From our findings, we observed overall improvement in glycemic control (OGTT, FBG, and HbA1c) in diabetic rats treated with the isoflavone-rich extract, in a dose-dependent manner. Previous studies reported on the excellent ability of soy isoflavones to lower blood glucose level when administered to STZ- induced animals (Lee, 2006; Shim, Kim, Seo, & Lee, 2007). Contrastingly, authors found increased levels of serum insulin and GSH concentration, and reduction in blood glucose level and methylglyoxal concentration when diabetic rats were treated with high isoflavone dose (550 mg/kg), while at low treatment doses (of 120 mg/kg),no positive effect was observed (Lu et al., 2008). Similarly, authors found no positive influence on the glycemic control in T2DM postmenopausal women treated with isoflavone supplementation at 132 mg/day (González, Jayagopal, Kilpatrick, Chapman, & Atkin, 2007). Although authors reported that at low dietary doses (240, 480, and 1920 mg/100 g diet), isoflavone did not lower blood glucose levels in diabetic animals (Hsu, Chiu, & Yeh, 2003), other studies found that the treatment of STZ induced animals with soy isoflavones (at dose of ≤600 mg/kg diet) lowered blood glucose level (Lee, 2006; Shim et al., 2007). Authors found that the treatment of diabetic animals with genistein improved glycemic control (Rehman, Ali, & Akash, 2019), and the treatment of same animals with formononetin and biochanin A, both isoflavones, also decreased HbA1c level and improve overall glycemic control (Oza & Kulkarnia, 2018a, 2018b). It could be suggested

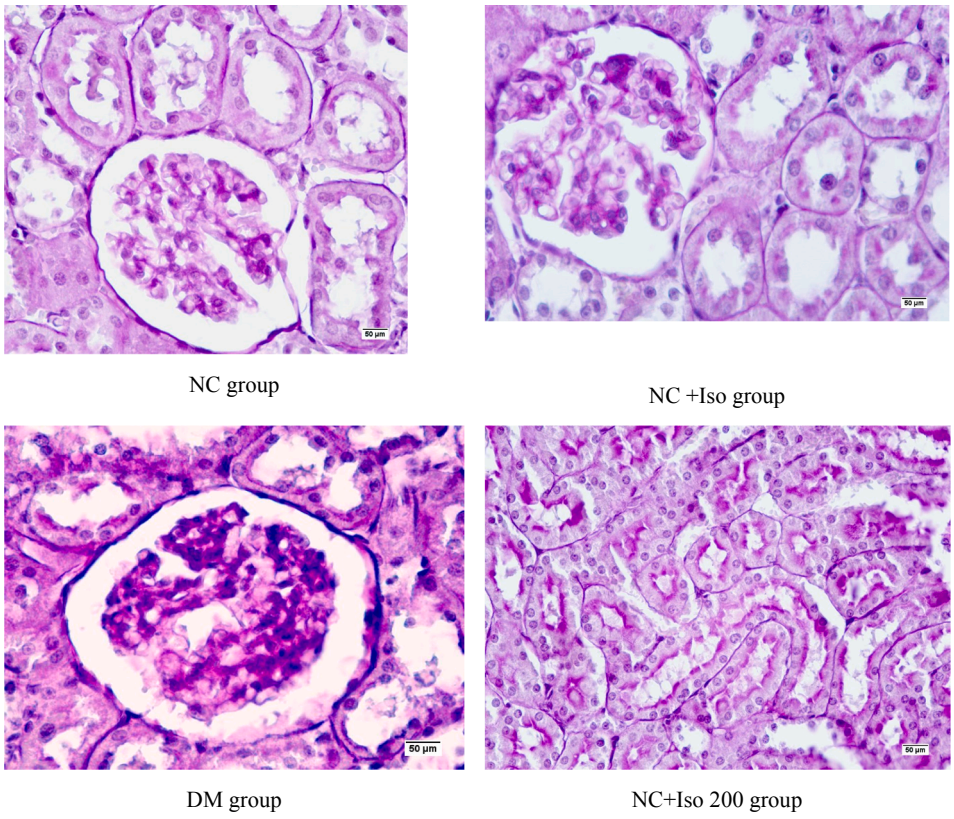

Fig. 5. Histological evaluation of renal tubules and renal corpuscle of experimental groups (×400 PAS staining). (a): NC group showing Normal epithelial cells and brush border of the proximal convoluted tubules; (b): NC + Iso group showing no nephrotoxic effect, normal epithelial cells and brush border of the proximal convoluted tubules; (c): DM group showing damage to the proximal convoluted tubules; (d): NC + Iso 200 group showing Normal cellularity of glomerulus, normal epithelial cells and brush border of the proximal convoluted tubules.

that the improvement in weight observed in diabetic rats treated with the isoflavone-rich extract might be a result of overall improvement in glycemic control.

Abnormal expression of liver enzymes such as, ALT, AST, and ALP has been associated with the manifestation of diabetes, thus level of these enzymes serve as vital indicators (Fernandes, Novelli, Fernandes Junior, & Galhardi, 2009; Tibi, Collier, Patrick, Clarke, & Smith, 1988). We observed that the elevated AST and ALT recorded in diabetic rats were lowered when treated with the isoflavone-rich kudzu extract at both doses. Similarly, authors reported that the oral administration of diabetic rats with biochanin A decreased the level of AST and ALT (Azizi, Goodarzi, & Salemi, 2014). The isoflavone-rich kudzu extract was also found not to negatively affect the activities of the liver enzymes in normal healthy rats. Interestingly, we found that serum level of ALP did not differ between experimental groups despite previous study reporting otherwise (Azizi et al., 2014). Authors also observed no significant difference between serum ALP level in diabetic and control subjects suggesting that there was no strong correlation between ALP levels and the manifestation of diabetes (Chen et al., 2017). However, it could be suggested that the short study duration could be the underlying reason for this effect.

Circulating urea and protein level are another valuable assessment tool in the diagnosis and management of diabetes (Chutani & Pande, 2017; Hasan & Abdulsattar, 2015; Xie et al., 2018). We found an overall improvement in both serum protein and urea level in diabetic rats treated with the isoflavone-rich kudzu extract compared to the untreated diabetic rats. Similarly, authors reported that the consumption of soy isoflavones decreased urinary albumin excretion and improved the total protein in diabetic nephropathy patients (Teixeira et al., 2004), and a 60 days treatment of insulin resistant rats with genistein decreased serum urea, and attenuated renal damage (Palanisamy, Viswanathan, & Anuradha, 2008). Also, it was reported that the treatment of STZ-induced diabetic rats with isoflavones-rich extract from *Pueraria tuberosa* tubers reduced level of serum urea (Tripathi, Shukla, Pandey, Pandey, & Kumar, 2017). We found no significant difference in serum protein and urea level in healthy rats and those treated with the isoflavone-rich extract. This could imply that the isoflavone-rich extract had no advance effect in protein metabolism even at high dose of 200 mg/kg.

Increase level of hematological indices such as WBC, MPV, PDW, PCT, and PLT count, have been implicated in diabetes complications (Demirtas et al., 2015; Kim et al., 2014; Twig et al., 2013). However, we found that WBC, MPV, and PDW did not significantly differs between diabetic and healthy rats. This contradicts previous study where significant difference in level of WBC, MPV, and PDW were reported between healthy and diabetic rats (Demirtas et al., 2015). It could be suggested that the study duration (28 days) was rather short to result in varying level of WBC, MPV, and PDW in experimental animals. However, we found an increase in PLT count and decrease in PCT levels in diabetic rats which were slightly ameliorated when treated with the isoflavones-rich extract. The isoflavones-rich extract had no advance effect on PLT count and PCT level in healthy rats. Authors found that supplementation of postmenopausal women's diet with soy showed no advance effect on PLT count and PCT level (Soung et al., 2006).

The loss of function and mass of pancreatic β-cells has been one of the major highlights in the pathogenesis of type 2 diabetes mellitus, making regeneration of these cells a possible therapeutic approach. We noticed that the treatment of diabetic rats with the isoflavone-rich extract at both doses stimulated the regeneration of β-cells, this was evident from the increase in the number of insulin and Ki-67 (proliferation marker) positive cells observed in DM and DM + Iso 200 group compared to the DM group. Authors reported that the treatment of diabetic rats with puerarin preserved pancreatic β-cell mass and maintained the pancreatic insulin content (Yaribeygi et al., 2018; Li et al., 2014; Yang & Kang, 2018). Previous study also found that the treatment of HFD and db/db mice with puerarin stimulated the proliferation of Ki-67/β-cells (Yang et al., 2016). Also, it was reported that the treatment of diabetic animals with formononetin (an isoflavone) ameliorated pancreatic tissue damage (Oza & Kulkarnia, 2018a).

Diabetes mellitus has been implicated as the most leading cause of

chronic renal disorders which usually manifests as histological changes of the kidney and alteration of its functions (Lim, 2014). Based on the histological evaluations of kidney tissues, we found that the isoflavones-rich extract can potentially ameliorate renal damage resulting from diabetes. Previously, authors found that treatment of diabetic animals with formononetin inhibited degenerative changes in kidney tissues (Oza & Kulkarnia, 2018a). Similarly study also that 8 weeks treatment of STZ-induced diabetic nephropathy mice with puerarin restored the glomerular structure and reduced dilatation of the renal tubules as compared to untreated diabetic animals (Xu et al., 2016).

Although this study was not aimed at comparing the antidiabetic properties of the isoflavone-rich extract obtained using different extracting solvents, it remains worthy to state that previous study found higher radical scavenging activity in Flos sophorae extract obtained using NADES compared with extract obtained using methanol (Nam et al., 2015). Despite a plethora of overwhelming preclinical findings, it was reported that 8 weeks treatment of Ovariectomized (OVX) mice with ethanol extract obtained from kudzu root showed no significant improvement on insulin secretion and serum leptin level, although blood glucose lowering effect was observed with the extract treatment (Tanaka et al., 2016). This could suggest that extraction solvent might have influence in the biological activities of different extracts. We highly recommend comparative preclinical studies devoted to elucidating the antidiabetic effect of isoflavone-rich extract obtained using different solvents.

5. Conclusion

The current study evaluates the anti-diabetic effect of isoflavone-rich extract obtained from dried kudzu root using natural deep eutectic solvent, and it was found that this extract reduced hyperglycemia and HbA1c level, enhanced glucose tolerance, and stimulated the regeneration of pancreatic β-cells. Furthermore, it was revealed that this isoflavones-rich extract can ameliorate potential liver injuries/damage and prevent possible alteration in histological structure and function of the kidney associated with diabetes pathogenesis. Additionally, we found no nephrotoxic effect when healthy animals were treated with this isoflavone-rich extract.

6. Ethics statements

All the animal protocols were approved by the ethical committee of institute of Immunology and Physiology of RAS, Ural branch.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2020.103922.

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